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Comparative Morphology of Chloroplasts in Podostemaceae Subfamilies Tristichoideae and Weddellinoideae suggests Evolution of Chloroplast Dimorphism

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Podostemaceae comprise a unique aquatic angiosperm family. Members of the family grow on rock surfaces of waterfalls and rapids in the tropics and subtropics. Recently, chloroplast dimorphism was reported for 13 species from the majority of clades in the subfamily Podostemoideae. Large chloroplasts with well-developed starch grains and small chloroplasts with few starch grains are located separately in the epidermal cells of roots and shoots. Transmission electron microscopy (TEM) analysis revealed that the large chloroplast is comparable to a typical, ordinary chloroplast, while the small chloroplast does not, but is specialized. To investigate whether chloroplast dimorphism is common in Podostemaceae, we conducted TEM and light microscopy of six species from two subfamilies, Tristichoideae and Weddellinoideae. All samples examined had uniform chloroplasts of the same size. Evaluation of their ultrastructure indicated they had normal grana and starch grains. These findings suggest that chloroplast dimorphism is a trait limited to Podostemaceae subfamily Podostemoideae.

Key words: Chloroplasts, Dimorphism, Evolution, Photosynthesis, Podostemaceae, TEM

The Podostemaceae are aquatic angiosperms of the tropics and subtropics and grow over rock surfaces in waterfalls and rapids. Podostemaceae comprise three subfamilies: Tristichoideae (6 genera, ca. 20 species), Weddellinoideae (1 genus, 1 species), and Podostemoideae, (47 genera, ca. 280 species) (Cook & Rutishauser 2007, Koi *et al.* 2012, Koi & Kato 2015). The plant body of members of the Podostemaceae is constantly submerged in rapidly flowing water during the rainy season, then emerge above the water surface to produce flowers and fruit in the dry season. To adapt to such a unique habitat, the Podostemaceae have evolved unique, unparalleled morphologies that deviate extremely from most angiosperms. The root is sometimes foliose, and functions both as an assimilatory organ and as an attachment organ to the rock surface (Rutishauser 1997, Rutishauser & Grubert 1999). Furthermore, in Podostemoideae the shoot appears to lack stems and comprises only leaves (Imaichi *et al.* 2005, Koi *et al.* 2005, Koi & Kato 2010).

Recently, we reported an interesting cytological characteristic in Podostemoideae, *i.e.*, the occurrence of distinctly dimorphic (large and small) chloroplasts in the epidermis of roots and leaves (Fig. 1, republished with Springer's permission, Fujinami *et al.* 2011). Transmission electron microscopy (TEM) of *Hydrobryum khaoyaiense* M. Kato showed that the large chloroplasts have well-developed starch grains like ordinary chloroplasts, while the small chloroplasts have very

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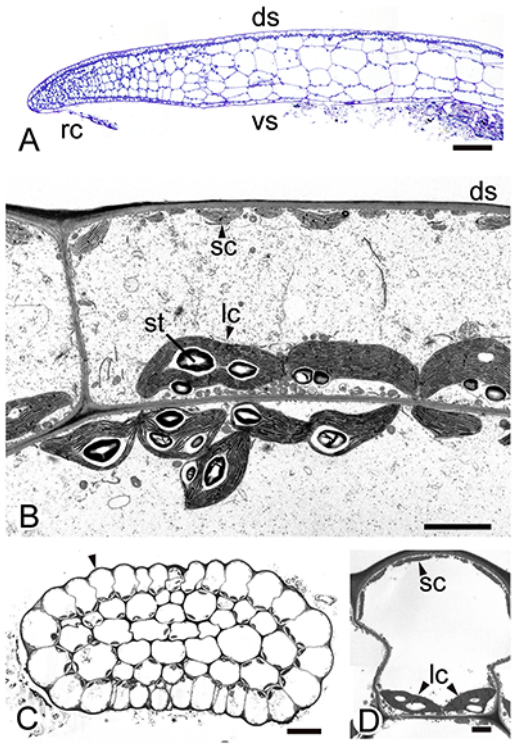

FIG. 1. *Hydrobryum khaoyaiense* (Podostemoideae), republished with Springer's permission (Fig. 2 in Fujinami *et al.* 2011). A. Light micrograph of radial longitudinal section of root with root apical meristem. B. Transmission electron microscopy image of dorsal epidermis and parenchyma of root. Small and large chloroplasts are arranged along outer and inner tangential walls of epidermal cells, respectively. C. Cross section of leaf. D. Epidermal cell of leaf. ds, dorsal side; lc, large chloroplast; rc, root cap; sc, small chloroplast; st, starch grain; vs, ventral side. Scale bars = 500 μm (A), 5 μm (B), 20 μm (C), and 2 μm (D).

few starch grains, although they show normal granum ultrastructure with three or four thylakoids per granum. The large and small chloroplasts are located separately in each epidermal cell along the inner and upper tangential cell walls (Fig. 1, Fujinami *et al.* 2011). To the best of our knowledge, this is a novel finding in angiosperms. We considered chloroplast dimorphism to be an adaptive structure for Podostemaceae where the small chloroplast may function to facilitate CO_2 uptake for photosynthesis in torrential water (Fig. 1, Fujinami *et al.* 2011). In addition to *H. khaoyaiense*, we confirmed the presence of similar chloroplast dimorphism in 12 species in 10 genera from the subfamily Podostemoideae using light microscopy (Fujinami *et al.* 2011). Each of these species belongs to a clade in the phylogenetic tree of Podostemoideae as previously published by Koi *et al.* (2012) (Fig. 5). Notably, for those 13 species, dimorphic chloroplasts in the epidermis have always been observed. Furthermore, there are no published reports of chloroplast dimorphism in the remaining two subfamilies, Tristichoideae and Weddellinoideae.

In this article, we report on TEM and light microscope (LM) studies of five species of subfamily Tristichoideae. Additionally, *Weddellina* (Weddellinoideae) was observed using LM. Based on our findings, we discuss the evolution of dimorphic chloroplasts in Podostemaceae.

Materials and Methods

The species examined, their collection sites, and the organs observed are listed in Table 1. Six species were selected as representatives of five genera of Tristichoideae and one genus of Weddellinoideae. Vouchers were deposited in the Forest Herbarium, Bangkok, Thailand (BKF) and the herbarium of the Department of Botany, the National Museum of Nature and Science, Tsukuba, Japan (TNS). Of the six species, *Terniopsis minor* M. Kato & Koi and *Dalzellia ubonensis* M. Kato were observed by TEM; the other four were observed by LM.

For TEM analysis, the collected materials were immediately immersed in a fixative solution of 1.6% glutaraldehyde (GA) in river water in the field to prevent chloroplast movement in response to changing light directions. The specimens in GA were kept at 4°C in a cool box for 24 hours, then post-fixed in 1.0% osmium tetroxide in 0.05 M phosphate buffer (pH 7.2) for 1 hour at 4°C. They were then dehydrated using an ethanol series, embedded in epoxy resin (Plain Resin; Nissin EM, Tokyo, Japan), and sectioned. Ultrathin sections (70 nm thick) were stained with uranyl

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TABLE 1. Materials examined and collection localities.

Species	Source	Organs of TEM*/LM** observation
Subfamily Tristichoideae		
Terniopsis minor M. Kato & Koi	Khlong Yai, Pong Nam Ron, Chanthaburi, Thailand; *S. Koi, R. Fujinami* & *T. Wongprasert TKF-104*	Roots, Stems, Leaves (TEM, LM)
Tristicha trifaria (Bory ex Willd.) Spreng.	Mawonge River at Ebone, Knongsanmba, Cameroon; *R. Imaichi, Y. Kita & J.-P. Ghogue CMR-29*	Roots, Stems, Leaves (LM)
Indodalzellia gracilis (Mathew, Jäger-Zürn & Nileena) Koi & M. Kato	Punnavoorthode River in Kerarla, India; *S. Koi & A. K. Pradeep SK-05*	Roots, Stems, Leaves (LM)
Dalzellia ubonensis M. Kato	Kaeng Lamduan stream, Yoddome Wildlife Sanctuary, Ubon Ratchathani, Thailand; *S. Koi, R. Fujinami, N. Katayama & T. Wongprasert TKF-15*	Roots, Stems, Leaves (LM)
Indotristicha ramosissima (Wight) P. Royen	Cheenganni Puzha near Iritti, Kannur, Kerala, India; *M. Kato* & *R. Imaichi KI-26*	Roots, Stems, Leaves (LM)
Subfamily Weddellinoideae		
Weddellina squamulosa Tul.	Head fall, Essequibo river, Guyana; *M. Kato, H. Okada, R. Imaichi, Y. Kita* & *K. Suzuki GU-03*	Roots, Stems, Leaves (LM)

*TEM: Transmission electron microscopy, **LM: Light microscopy.

acetate and lead citrate and observed using a JEM 1200-EX (JEOL, Tokyo, Japan). For LM analysis, the collected materials were fixed in formalin/acetic acid/50% ethanol (5/5/90 v/v/v) in the field. Fixed materials were dehydrated with a graded series of ethanol, embedded in HistoResin (Glycol methacrylate, Leica, Heidelberg), cut into 2 μm-thick sections, and stained with modified Sharman's staining solution (Jernstedt *et al.* 1992).

To compare the size of chloroplasts from *Terniopsis minor* and *Dalzellia ubonensis*, we measured the area of each chloroplast cut through the median sagittal plane from the TEM images of one section using a microanalyzer (Nihon Poladigital Co., Tokyo, Japan).

Results

TEM observations of Terniopsis minor

The plant body of *Terniopsis minor* comprises creeping roots and shoots arising from the roots (Fig. 2A, B). The epidermal cells of the roots containing chloroplasts of similar size and ultrastructure are located along the radial and ventral cell walls (Fig. 2C). Even in immature cells of the root apical meristem (Fig. 2D), the chloroplasts in cells on the dorsal surface were also isomorphic (Fig. 2E), although they were smaller than the chloroplasts in the differentiated epidermal cells. In ultrastructure, the chloroplasts in the epidermis had normal grana and starch grains, as did chloroplasts in the parenchyma (Fig. 2C). The area in the cross section of the chloroplasts in the epidermis was 1.7 ± 0.8 μm^2 (n = 23 chloroplasts from 15 epidermal cells), and 2.2 ± 1.0 μm^2 (n = 14 chloroplasts from 7 cells) in the parenchyma (Fig. 2C). Thus, the chloroplasts in the epidermis are comparable to those in the parenchyma.

The leaf cells from *Terniopsis minor* similarly have uniform chloroplasts, although the chloroplasts from the leaf (area = 6.5±1.4 μm^2, n = 36 chloroplasts from 10 cells) were larger than those observed in the roots (Fig. 2F, G). The epidermal and inner cells of the midrib also contain uniform chloroplasts, although the inner cells have smaller chloroplasts than the surface cells (Fig. 2H). In ultrastructure, the chloroplasts from the leaf tissue also contained normal grana and starch grains (Fig. 2I). The epidermal cells of the stem also had uniform chloroplasts (data not shown).

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TEM observations of Dalzellia ubonensis

The plant body of *Dalzellia ubonensis* lacks roots and consists of only foliose stems and small leaves (Fig. 3A). The dorsal epidermis of the foliose stem had chloroplasts of the same size, indicating a lack of chloroplast dimorphism (Fig. 3B, C). Our TEM analysis revealed that those isomorphic chloroplasts have normal grana and starch grains (Fig. 3D). The area of these chloroplasts was 2.1 ± 0.5 μm^2 (n = 25 chloroplasts from 15 cells), which was similar in area to the chloroplasts located in the parenchyma (2.7 ± 1.8 μm^2, n = 9 chloroplasts from 5 cells). The immature surface cells near the apical meristem of the stem also contained uniform chloroplasts, although they were smaller (2.0 ± 0.6 μm^2, n = 20 chloroplasts from 10 cells) than those of the differentiated epidermal cells (arrow in Fig. 3B, E).

In leaf cells, the chloroplasts were similar in size, with areas of 12.3 ± 3.1 μm^2 (n = 33 chloroplasts from 7 cells) (Fig. 3F, G). These chloroplasts had normal grana and contained some starch grains (Fig. 3G).

LM analysis of several genera of Tristichoideae and one genus of Weddellinoideae

The plant bodies of several genera of Tristichoideae and *Weddellina* of Weddellinoideae are similarly constructed, with creeping roots and shoots. In *Indotristicha ramosissima* (Wight) P. Royen, the stem and leaf epidermis had uniform chloroplasts that were similar in size to those of the inner tissue of the stem and the midrib (Fig. 4A, B). In *Tristicha trifaria* (Bory ex Willd.) Spreng. and in *Indodalzellia gracilis* (Mathew, Jäger-Zürn & Nileena) Koi & M. Kato, the leaf epidermis had uniform chloroplasts (Fig. 4C, D). For the two species, the stem epidermis also had chloroplasts that were uniform in size (data not shown).

In *Weddellina squamulosa* Tul. (subfamily Weddellinoideae), the chloroplasts from the epidermis of the roots, stems, and leaves were uniform, indicating a lack of chloroplast dimorphism (Fig. 4E–H).

Discussion

Fujinami *et al.* (2011) previously suggested the ecophysiological function of small chloroplasts in the epidermis of Podostemoideae. The epidermis with dimorphic chloroplasts is subjected to torrential water. Submerged freshwater angiosperms, for example, *Potamogeton lucens* L., *Elodea canadensis* Michx., *Myriophyllum spicatum* L., and *Hydrilla verticillata* (L.f.) Royle, utilize HCO_3^- as a source of CO_2 for photosynthesis. Active HCO_3^- uptake is performed through a HCO_3^- pump located at the plasmalemma (Prins *et al.* 1982, Salvucci & Bowes 1983). The small chloroplasts in the epidermis of Podostemoideae probably function in CO_2 uptake from torrential water through a HCO_3^- pump located at the plasmalemma, which may be an energy-consuming process (Prins *et al.* 1982, Salvucci & Bowes 1983, Fujinami *et al.* 2011). The small chloroplasts of Podostemaceae may therefore supply the energy for the HCO_3^- uptake process.

In five species of Tristichoideae (*Terniopsis minor, Tristicha trifaria*, *Indodalzellia gracilis, Dalzellia ubonensis*, and *Indotristicha ramosissima*) and one species (*Weddellina squamulosa*) of the Weddellinoideae, chloroplasts in the epidermal cells did not show dimorphism. TEM observations of *T. minor* and *D. ubonensis* indicated that those isomorphic chloroplasts had normal grana and well-developed starch grains and were similar in size and ultrastructure to the chloroplasts in the parenchyma cells. We therefore conclude that the epidermal cells from the five species of Tristichoideae and one species of Weddellinoideae did not have small chloroplasts comparable to those observed in Podostemoideae (Fujinami *et al.* 2011).

Thirteen species (*Cladopus japonicas* Imamura, *Dicraeanthus africanus* Engl., *Endocaulos mangorense* (Perr.) C. Cusset, *Hydrobryum japonicum* Imamura, *Hydrobryum khaoyaiense*, *Macropodiella heteromorpha* (Baill.) C. Cusset, *Macropodiella pellucida* (Engl.) C. Cusset, *Marathrum schiedeanum* (Cham.) Tul., *Mourera fluviatilis* Aubl., *Rhyncholacis* sp., *Thelethylax*

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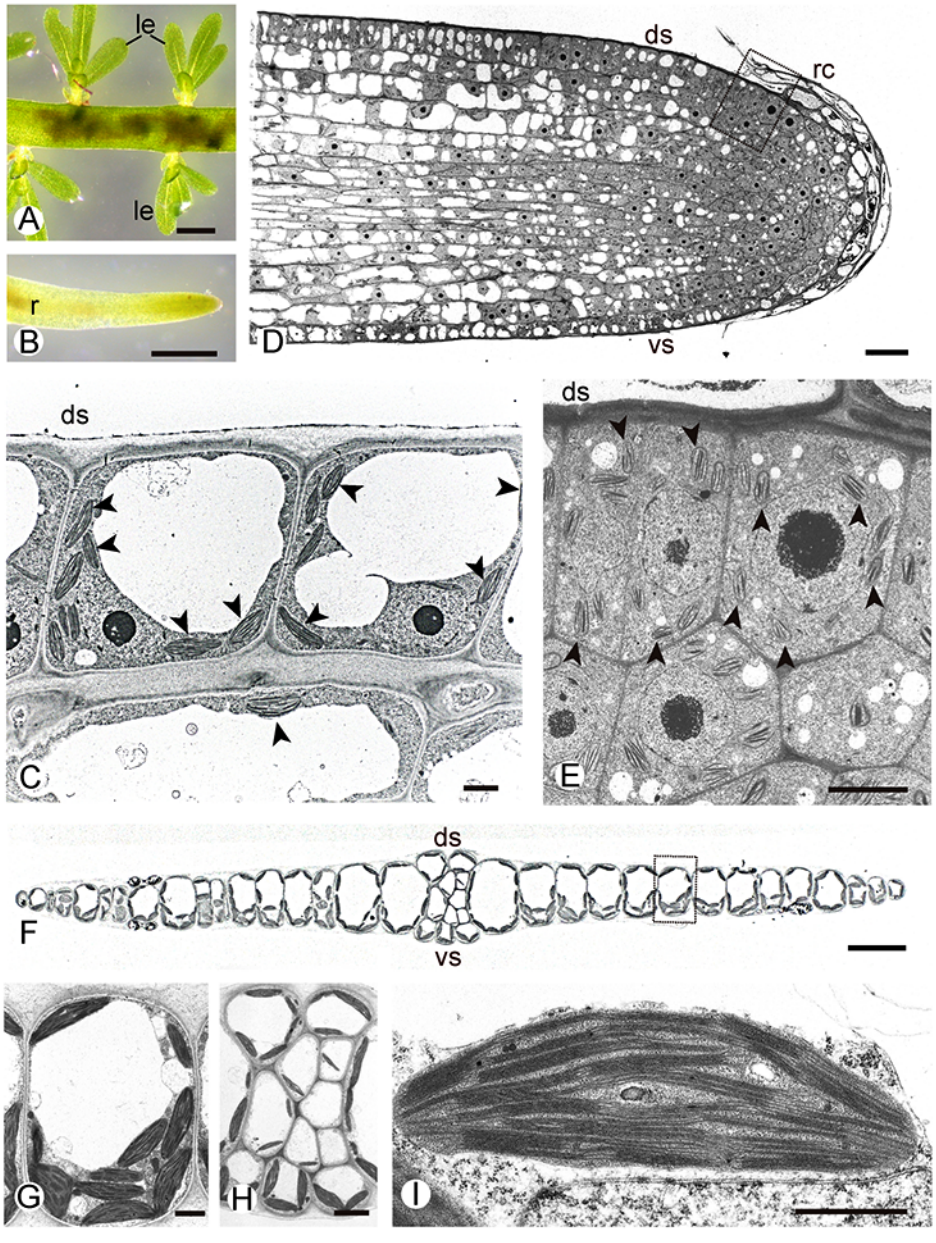

FIG. 2. *Terniopsis minor* (Tristichoideae). Stereomicroscopy (A, B) and transmission electron microscopy (C–I) images. A. Shoot-bearing root. B. Root tip. C. Dorsal epidermis and parenchyma cells of root, 1.5 mm behind anterior end with isomorphic chloroplasts (arrowheads). D. Radial longitudinal section of root tip. Square area is magnified in E. E. Dorsal surface cells of root apical meristem, with younger chloroplasts (arrowheads) than chloroplasts of C. F. Cross section of mature leaf. Rectangle is magnified in G. G. Leaf cell. H. Midrib of leaf. I. Magnified figure of chloroplast in leaf. ds, dorsal side; le, leaf; r, root; rc, root cap; vs, ventral side. Scale bars = 1 mm (A and B), 2 µm (C, G), 20 µm (D, F), 5 µm (E, H), and 1 µm (I).

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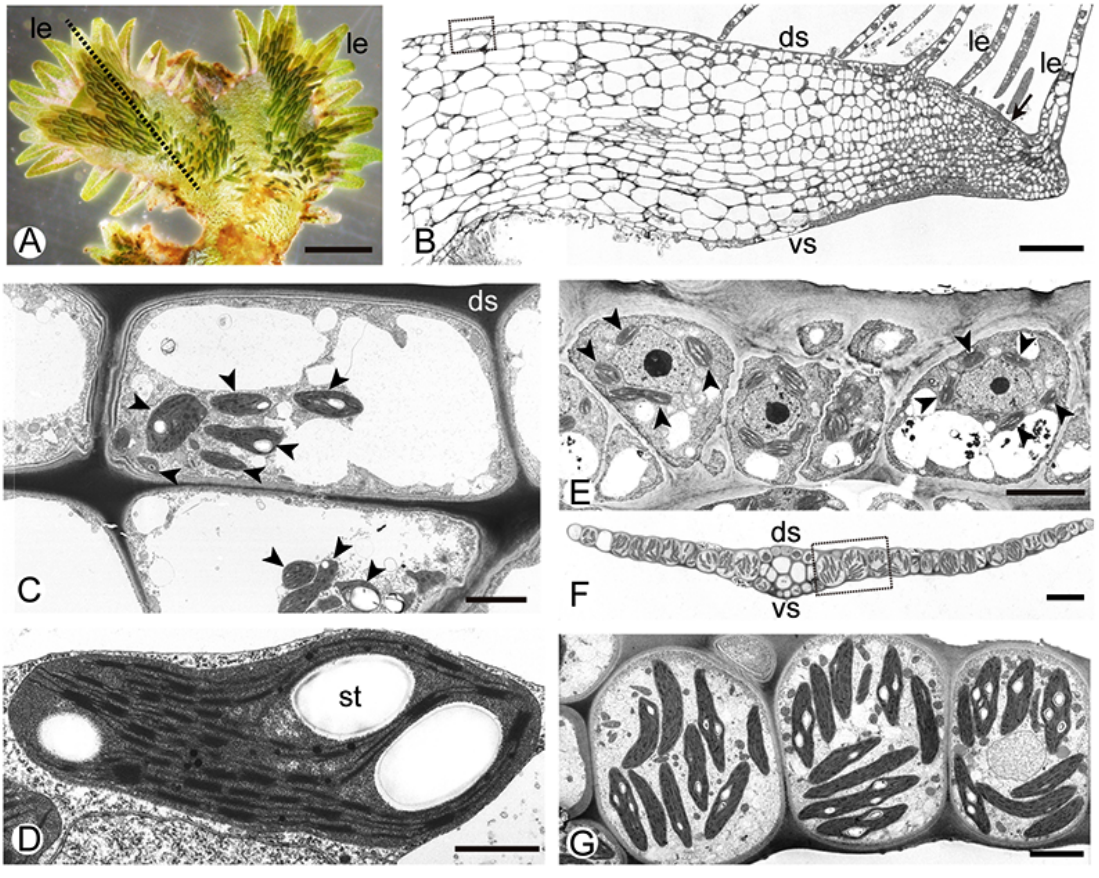

FIG. 3. *Dalzellia ubonensis* (Tristichoideae). Stereomicroscopy (A) and transmission electron microscopy (B-G) images. A. Foliose shoot with dorsal and marginal leaves. Broken line indicates orientation of longitudinal section, which is shown in B. B. Longitudinal section of foliose shoot with shoot apical meristem. Square area is magnified in C. Arrow indicates surface cells near apical meristem. C. Dorsal epidermal cells and parenchyma cells with isomorphic chloroplasts (arrowheads). D. Closeup of chloroplast of dorsal epidermal cell. E. Surface cells near apical meristem indicated in B. F. Cross section of young marginal leaf. Square is magnified in G. G. Leaf cells. Uniform chloroplasts are found in each leaf cell. ds, dorsal side; le, leaf; st, starch grain; vs, ventral side. Scale bars = 1 mm (A), 100 µm (B), 5 µm (C, E, G), 1 µm (D), and 20 µm (F).

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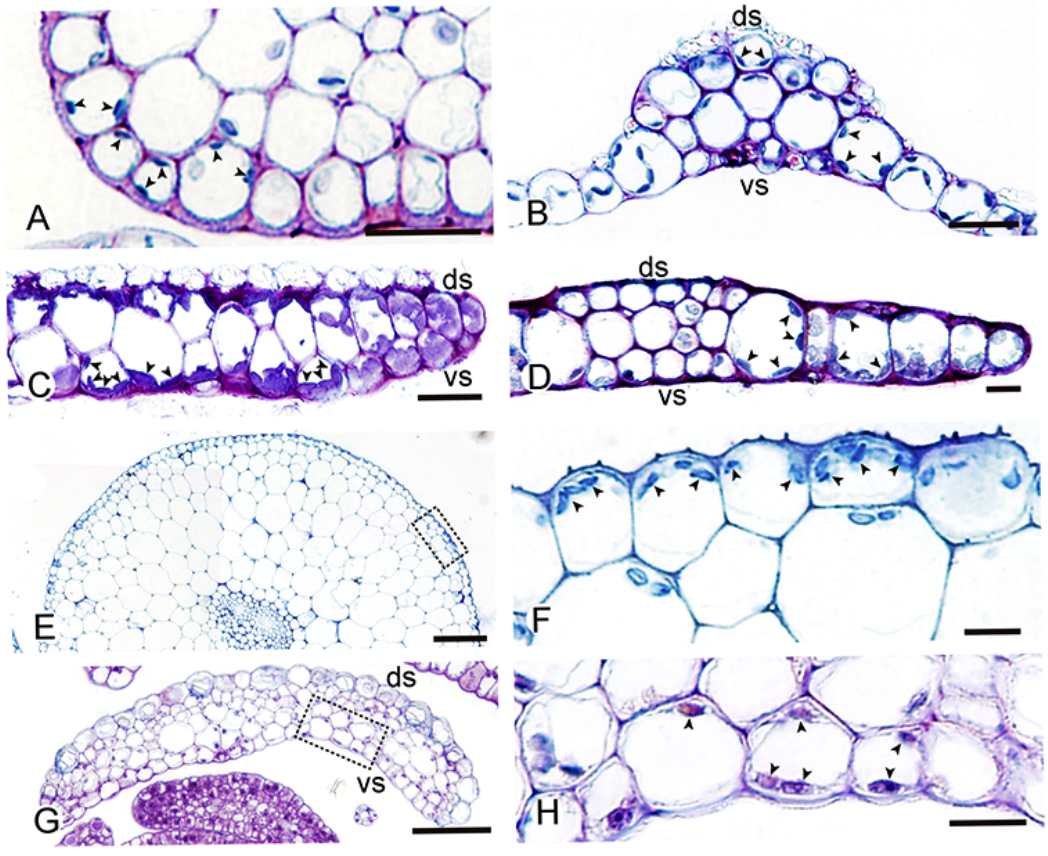

FIG. 4. Three species of Tristichoideae and *Weddellina squamulosa* (Weddellinoideae).Cross section of stem (A, E, F) and leaf (B-D, G, H) using LM. Arrowhead indicates uniform chloroplast in epidermal cells of stems and leaves. A-B. *Indotristicha ramosissima*. A. Epidermis of stem. B. Midrib and laminae of leaf. C. *Tristicha trifaria*. D. *Indodalzellia gracilis*. E-H. *Weddellina squamulosa*. E. Stem from mature plant. F. Magnified image of stem in E. Uniform chloroplasts were found in epidermal cells. G. Mature leaf. H. Magnified image of leaf in G. ds, dorsal side; vs, ventral side. Scale bars: 20 µm (A-C), 10 µm (D, F, and H), 100 µm (E), and 50 µm (G).

minutiflora (Tul.) C. Cusset, *Zeylanidium lichenoides* Engl., and *Zeylanidium subulatum* (Gardner) C. Cusset) from 10 genera and 4 of the 15 clades of Podostemoideae (Koi *et al.* 2012) have dimorphic chloroplasts in the epidermis (Fujinami *et al.* 2011); a relatively small number out of the 280 species in this subfamily (Fig. 5, Koi *et al.* 2012). We noted, however, that there were no exceptions to chloroplast dimorphism in the 13 species examined, which belong to four phylogenetically scattered clades. Taking this findings into account, we hypothesize that the small chloroplasts might have been acquired when the subfamily Podostemoideae diverged (Fig. 5).

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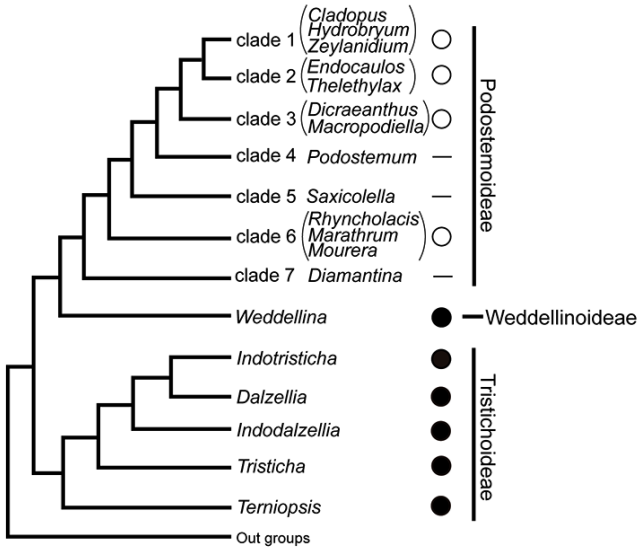

FIG. 5. Character states of dimorphic chloroplasts mapped onto phylogenetic tree of Podostemaceae (modified from Koi *et al*. 2012). Genus names presented in clades 1–3 and 6 of Podostemoideae derived from previous studies. Open circles indicate presence of dimorphic chloroplasts in epidermal cells. Solid circles indicate presence of isomorphic chloroplasts (i.e., absence of chloroplast dimorphism) in epidermal cells. Bar indicates unobserved taxa, *Podostemum* (clade 4), *Saxicolella* (clade 5), and *Diamantina* (clade 7).

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